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EXPERIMENTAL STUDIES

Dissolution of Thrombotic Arterial Occlusion by High Intensity, Low Frequency Ultrasound and Dodecafluoropentane Emulsion: An In Vitro and In Vivo Study

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Objectives. **We examined the effectiveness of the microbubbles of an echo contrast agent, dodecafluoropentane (DDFP) emulsion, to enhance low frequency ultrasound clot disruption in vitro and in vivo.**

Background. **Ultrasound is reported to facilitate clot dissolution, and microbubbles could theoretically enhance ultrasound clot dissolution by augmenting cavitational effects.**

Methods. In vitro studies**: The disruption rate of fresh human clots by ultrasound (24 kHz, 2.9 W/cm^2) was examined in saline and DDFP emulsion. *In vivo studies*: Using a rabbit iliofemoral thrombotic occlusion model, recanalization rate and histopathologic findings were compared among groups treated with DDFP emulsion alone, transcutaneous ultrasound (20 kHz, 1.5 W/cm^2) alone and with DDFP emulsion and ultrasound combined.**

Results. **The ultrasound clot disruption rate was significantly ($p < 0.01$) increased, from 72 ± 18% (mean ± SD) in saline to 98 ± 4% in DDFP emulsion in 3 min in vitro. No vessel was recanalized by DDFP emulsion alone (0%), and only a single artery was patent after ultrasound treatment alone (9%). In contrast, 82% of iliofemoral arteries were angiographically recanalized after ultrasound treatment with DDFP emulsion. Histologically, the patent arteries had only minimal focal mural thrombus, with no evidence of vessel wall damage. However, substantial damage was observed in rabbit dermis and subcutaneous tissue.**

Conclusions. **1) DDFP emulsion, an echo contrast agent, significantly enhances the clot-disrupting effect of low frequency ultrasound in vitro and in an in vivo rabbit iliofemoral occlusion model. 2) This simple combination therapy has potential for clinical application in patients with thrombotic arterial occlusions.**

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Thrombolytic therapy is the most widely used technique to recanalize the abruptly occluded coronary artery in patients with myocardial infarction. Although intravenous thrombolysis reduces mortality (1–5) and preserves left ventricular function (3,6,7), thrombolytic agents have several unresolved problems: 1) The initial recanalization rate is ~70%; 2) Thrombolysis in Myocardial Infarction (TIMI) grade 3 flow is achieved in <60% of patients; 3) intermittent patency and reocclusion of the recanalized artery are not uncommon (8); 4) hemorrhagic complications are more frequent at higher dosages and in older patients; and 5) substantial time is necessary to achieve successful recanalization. Therefore, the development of a new noninvasive technique that can rapidly dissolve blood clots without an incremental hemorrhagic risk would be very useful.

Application of ultrasound energy for clot dissolution has been investigated for >20 years. Catheter-delivered low frequency (17.5 to 27.5 kHz) ultrasound had succeeded in dissolving clots in vitro (9–15), in vivo in animal models (12,14,16) and in patients (16,17) without thrombolytic agents. However, like other catheter-based interventional techniques, this technique is limited by the time delay to intervention and by other technical, financial and logistic factors. Transcutaneous exposure of ultrasound energy has been reported to enhance the effect of thrombolytic agents in vitro (18–27) and in vivo in animal models (19,28–30). However, relatively few studies have examined the clot-disrupting effects of transcutaneous ultrasound energy without thrombolytic agents (26,27,31).

The most likely mechanism of low frequency ultrasound clot disruption without the concomitant use of a thrombolytic agent is reported to be acoustic cavitation (unstable or transient cavitation), which is the generation, growth and disrup-

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Abbreviations and Acronyms
DDFP = dodecafluoropentane
TIMI = Thrombolysis in Myocardial Infarction

tion of microbubbles (9,10,13–15,19,26,27,30–33). When the bubbles collapse, a very high local pressure charge is produced. Another possible mechanism is precavitation (stable cavitation), which is the oscillation or vibration of microbubbles (20,21,29). Precavitation itself produces large shock waves and also causes microstreaming of liquids. Recently, various transpulmonary echo contrast agents, which are basically microbubbles, have been developed to opacify the left ventricular cavity and even to illustrate myocardial perfusion by intravenous injection (34–38). Tachibana and Tachibana (39) demonstrated that sonicated human serum albumin microbubbles enhanced in vitro thrombolysis with combined application of urokinase and ultrasound (170 kHz). Porter et al. (27) reported the efficacy of perfluorocarbon-exposed sonicated dextrose albumin in enhancing ultrasound clot disruption in vitro.

Therefore, we hypothesized that the addition of microbubbles, which have been used as transpulmonary echo contrast agents, might enhance lower frequency ultrasound clot disruption by increasing the number of microbubbles and the energy produced by collapse or oscillation of microbubbles, or both.

Accordingly the aims of this study are 1) to examine the effect of the microbubbles of a transpulmonary echo contrast agent on ultrasound clot disruption in vitro, and 2) to investigate the enhancing effect of microbubbles on the in vivo recanalization rate of thrombotically occluded rabbit iliofemoral arteries by transcutaneous ultrasound exposure.

Methods

In vitro study. *Clot preparation.* Whole blood was obtained by antecubital venipuncture from three healthy volunteers in the morning after a 12-h fast. The blood was allowed to coagulate in a glass test tube at room temperature for 2 to 3 h. After serum was aspirated, the clots were cut into pieces 417 to 543 mg in weight. Each clot was weighed on a precision scale (Mettler PN 323) and placed in a plastic cassette (33 × 28 × 6 mm) with 118 rectangular windows (5 × 1 mm), usually used for processing pathologic specimens, to protect each clot from the potential thrombus-disrupting effects of floating under ultrasound exposure and to keep the clot at the same site in the ultrasound field.

In vitro ultrasound system. The in vitro ultrasound system consists of an ultrasound generator (ENI generator EGR-700, Blackstone Ultrasonics) and a transducer beaker of 304 stainless steel, with a capacity of 600 ml (84-mm diameter, 109-mm height, 0.8-mm wall thickness). The transducer is located on the outside bottom center of the beaker. This system operates in continuous mode at a power output of 50 W with a frequency of 24.8 kHz. The calculated acoustic intensity was 2.93 W/cm^2, and the ultrasound pressure level was 42.7 psi. The ultrasound field produces plane waves in the liquid, and no dissipation of ultrasound energy was expected at the site of thrombi, which were located ≤4 mm above the transducer.

Echo contrast agent. An echo contrast agent, EchoGen (Sonus Pharmaceuticals, Inc.), which is under clinical investigation, was used to examine its enhancing effect of ultrasound clot disruption. EchoGen is a 2% dodecafluoropentane (DDFP) emulsion that makes a phase shift at body temperature from liquid to microbubbles (34,35,37,38). DDFP is usually injected intravenously to opacify the left side of the heart, is mainly excreted from the lung, and it has the following pharmacokinetics in blood (one-compartment model): half-life in beta-phase 2.45 ± 0.26 min (mean ± SD), area under curve 19.3 ± 2.4 ppm·min; and total clearance 3,885 ± 465 liters/min/per kg (unpublished data, 1995). Three ml of DDFP were diluted with 1,000 ml of warmed saline (0.3 vol%), and appropriate amounts of this solution were used to fill the transducer beaker. Assuming that the blood pool of the average human is ~8% of body weight and blood plasma occupies 50% to 60% of total blood volume, the simulated dosage in humans was calculated to be 0.12 to 0.14 ml/kg. DDFP was used in an animal model in a wide range of doses from 0.01 to 1.0 ml/kg, and the dosages used in human clinical trials were 0.01 to 0.1 ml/kg (unpublished data, 1995).

Clot disruption protocol. Four groups of clots, each of which consisted of eight clots, were examined. The first two groups of clots, each of which was in a plastic cassette, were placed in degassed saline or DDFP and incubated at 37°C for 3 min without exposure to ultrasound. The second two groups of clots, each of which was in a plastic cassette, were also placed in each solution warmed to 35°C, respectively, and exposed to 24.8 kHz of ultrasound for 3 min. The temperature of the solution exposed to ultrasound increased up to 37° to 38°C after 3 min. After external ultrasound exposure, each clot was weighed on the specimen scale again. Absolute reduction and percent reduction in clot weight after each procedure were calculated as the extent of clot disruption.

Measurement of particulate size. The solution containing the dissolved clot was collected after each procedure, and a flow cytometric analyzer (Technicon H-I, Instruments Corp.) was used to measure the size of particulate debris after ultrasound clot disruption. The details of the method used in our laboratory have been previously reported (14). Particulate size was calculated using the formula $V = \pi d^3/6$, where d = diameter; and V = volume; and assuming that the particulate can be approximately spherical. The validity of this spherical approximation and the implication of other shapes have previously been reported by our laboratory (12). DDFP droplets were not included in the particulate size measurement because at room temperature they are too small (~0.3 to 0.4 μm in diameter) to be detected by this technique.

In vivo study. *Induction of thrombotic occlusion.* American Physiological Society Guidelines for Animal Research were followed, which conform to the "Position of the American

Heart Association on Research Animal Use" adopted by the Association in November 1984. Nineteen adult New Zealand White rabbits weighing 3.4 to 5.1 kg were anesthetized and maintained with ketamine (20 mg/kg body weight) and xylazine (3.0 mg/kg) intravenously. A 5F arterial sheath was inserted by cutdown surgical technique into the right carotid artery. After sheath insertion, 1 rabbit died of aortic dissection, and 18 rabbits (4.2 ± 0.5 kg) were included in this study.

Thrombotic occlusion in the iliofemoral artery was induced by electricity, as previously described by our laboratory (14). A 3.5F coronary Tracker catheter over a 0.014-in. coronary guide wire was inserted through the carotid arterial sheath into the iliofemoral artery. The guide wire was advanced to protrude 1 cm beyond the tip of the Tracker catheter. Subsequently, a 3-V battery was connected with its positive end to the wire and with the negative end to the rabbit's skin. Electrical interference on the electrocardiographic monitor indicated that an electric current was established. Complete occlusion of the iliofemoral artery was confirmed by angiography, which was performed every 30 min after the initiation of electrical stimulation. Each angiogram was acquired just after intraarterial injection of 100 μg of nitroglycerin to exclude possible arterial spasm. Accordingly, the age of the in vivo clots after complete arterial occlusion was <30 min.

Experimental protocol 1: ultrasound alone versus ultrasound and DDFP combined. Twenty-four iliofemoral arteries of 12 rabbits were used to compare the in vivo enhancing effects of DDFP on ultrasound clot dissolution. This experiment was performed in two randomly allocated consecutive phases: one phase for transcutaneous ultrasound alone on an occluded iliofemoral artery and the other for both transcutaneous ultrasound and DDFP administration on the contralateral side. One rabbit died after completion of the phase for transcutaneous ultrasound alone; therefore, 22 thrombotically occluded iliofemoral arteries of 11 rabbits (mean weight 4.0 ± 0.5 kg, range 3.4 to 5.1) were included in this protocol.

At the phase for combined treatment with transcutaneous ultrasound and DDFP, ultrasound was applied transcutaneously over the area of the thrombotically occluded artery through ultrasound gel after injection of 2 ml (initial dosage) of DDFP (nondiluted EchoGen) at room temperature into the iliac artery on the occluded side. The arterial occlusion site was identified by a metallic marker placed on the skin. It was placed above the occlusion site at the time of the angiogram that documented the induction of the arterial occlusion. Angiography was performed after 10 min of ultrasound exposure. If TIMI grade 3 flow was achieved, transcutaneous ultrasound treatment was terminated. If TIMI grade 0 to 2 arterial flow remained, transcutaneous ultrasound exposure was continued for 10 more min after intraarterial injection of 1 ml (additional dosage) of DDFP, and angiography was performed. This procedure was repeated maximally four times or until TIMI grade 3 flow was achieved. Therefore, at maximum, the total dosage of DDFP used was 5 ml, and the total time period of transcutaneous ultrasound exposure was 40 min with a 60-min postocclusion period that included 20 min of intermissions (5 min × 4) for DDFP emulsion injection and angiography. To prevent thermal damage to the skin by ultrasound, ultrasound gel placed between the transducer and the skin was cooled by ice, and during 5 min of intermission, the transducer was cooled by ice and the skin was cooled by cold water or cooled gel.

At the phase for transcutaneous ultrasound treatment alone on the contralateral side, the same procedure was performed, except for the intraarterial injection of DDFP. If the phase for transcutaneous ultrasound alone was initially allocated, no part of the thrombus occluding the artery was exposed to DDFP. Even when this phase was accomplished after the combined treatment with transcutaneous ultrasound and DDFP, previously injected DDFP was considered to have disappeared from the cardiovascular system of the rabbit because its in vivo half-life is ~2 to 3 min, and 90 to 180 min was required to induce a new iliofemoral thrombotic occlusion on the contralateral side. At 15 and 30 min after the termination of each phase, angiography was repeated to evaluate the patency and TIMI grade flow of the artery exposed to transcutaneous ultrasound.

In four rabbits, to examine the pure thermal effect of ultrasound, tissue temperatures of the skin at which the ultrasound probe was attached and the soft tissue around the iliofemoral artery were measured with a needle thermal probe (Omega Engineering, Inc.) without any cooling procedures before and 5 and 10 min after ultrasound exposure.

Experimental protocol 2: DDFP alone versus ultrasound and DDFP combined. To compare the direct thrombolytic and combined effects of DDFP with transcutaneous ultrasound, 12 iliofemoral arteries of six rabbits (mean weight 4.4 ± 0.5 kg, range 3.8 to 5.0) were used in this protocol. This experiment was also performed in two randomly allocated consecutive phases: one phase for injection of DDFP alone into a thrombotically occluded iliofemoral artery and the other for both transcutaneous ultrasound and DDFP administration on the contralateral side.

At the phase for combined treatment with transcutaneous ultrasound and DDFP, the procedure was exactly the same as that for experimental protocol 1. At the phase for DDFP administration alone, the occluded artery was not exposed to ultrasound, and DDFP was injected into the iliac artery proximal to the occluded site, as performed in the phase for combined treatment.

Transcutaneous ultrasound system. Transcutaneous ultrasound was exposed from a 0.5-in sonicating horn with a flat tip that was connected to a converter and a generator (Heat Systems—Ultrasonics, Inc.). This system operates in continuous mode at a frequency of 20 kHz and an intensity of 1.5 W/cm^2.

Angiographic procedure. Manual injection of 1 to 2 ml of Omnipaque (Sanofi Winthrop Pharmaceuticals) was used for each angiogram. All studies were recorded with both 35-mm cine film at 30 frames/s and digital acquisition (Advantex, DXC, GE Medical System) with a posteroanterior projection. The angiograms were analyzed by a consensus of four investi-

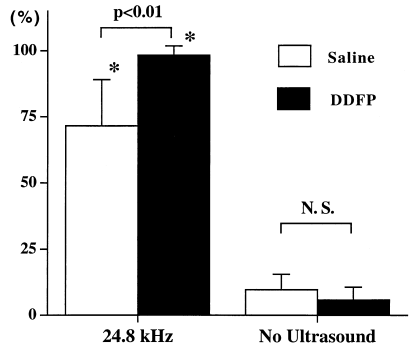

Figure 1. Effect of low frequency ultrasound, DDFP and combination of ultrasound and DDFP on percent reduction of clot weight. Clot weight reductions were significantly ($p < 0.01$) greater after low frequency ultrasound exposures than control without ultrasound. DDFP did not enhance clot weight reduction without ultrasound. However, DDFP combined with low frequency ultrasound significantly ($p < 0.01$) accelerated clot weight reduction from 72 ± 18% in saline to 98 ± 4%. *$p < 0.01$ versus no ultrasound with corresponding solution.

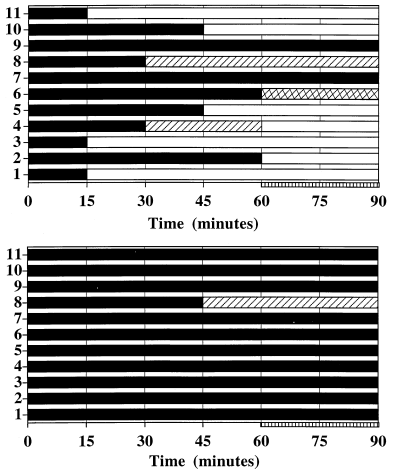

Figure 2. TIMI flow grade of arteries treated by ultrasound exposure and DDFP administration (**top**) and by ultrasound alone (**bottom**). Of the 11 iliofemoral arteries treated by combination of transcutaneous ultrasound and DDFP, 9 were recanalized (TIMI grade 3 flow **[open bars]** in 7, TIMI grade 2 flow **[hatched bars]** in 1, TIMI grade 1 flow in 1 **[crosshatched bar]**). Only one artery was recanalized with TIMI grade 2 flow after ultrasound exposure alone. **Solid bars** = TIMI grade 0 flow; **striped bar** = postintervention period.

gators (T.N., H.L., H.B., R.J.S.) for the presence or absence of occlusion, TIMI grade flow, vessel spasm and distal or side branch embolization.

Pathologic studies. After the experiment, 11 rabbits in protocol 1 were euthanized by injecting 3 to 4 ml of sodium pentobarbital intravenously. The iliofemoral arteries, ultrasound-exposed skin and soft tissues were excised, examined grossly and then fixed in 10% neutral buffered formalin for 24 to 72 h. The iliofemoral arteries were then cut transversely every 2 to 3 mm for the length of the vessel. Samples were dehydrated in graded alcohol, cleared in Hemo-De and embedded in paraffin. Sections 4 to 5 μm thick were cut and mounted on glass slides and stained with hematoxylin and eosin.

Statistical analysis. Results are given as mean value ± SD. In the in vitro study, percent reductions of clot weight after each procedure were compared using the unpaired Student *t* test. The Fisher exact test was performed to compare the results of in vivo ultrasound clot disruption rate with and without DDFP; $p < 0.05$ was considered statistically significant.

Results

In vitro study. *Effect of low frequency ultrasound exposure on clot disruption.* The percent clot weight reductions in saline were 10 ± 6% after 3 min of incubation without ultrasound and 72 ± 18% after 3 min of ultrasound exposure at 24.8 kHz, as shown in Figure 1. Clot weight reductions were significantly ($p < 0.01$) greater after ultrasound exposures.

Effect of microbubbles (echo contrast agent) on ultrasound clot disruption. Additional effects of the echo contrast agent DDFP on ultrasound clot disruption are shown in Figure 1. Without ultrasound exposure, the clots lost 10 ± 6% of their weight during 3 min of incubation in saline. DDFP did not enhance clot weight reduction (7 ± 5%) over saline control after 3 min of incubation without ultrasound.

After 3 min of ultrasound exposure, DDFP significantly ($p < 0.01$) accelerated clot weight reduction from 72 ± 18% in saline to 98 ± 4%, a nearly complete disruption of whole clots with a mean weight of 490 mg.

Particulate size after ultrasound clot disruption. The average diameter of clot debris after 3 min of ultrasound exposure was 3.3 μm (range 2.8 to 3.8) in saline and 3.6 μm (range 3.0 to 4.2) in DDFP emulsion. These particulate sizes are smaller than those of red blood cells.

In vivo study. *Angiographic results.* Angiography confirmed that all 34 iliofemoral arteries of 17 rabbits (11 in protocol 1, 6 in protocol 2) were occluded (TIMI grade 0 flow).

In protocol 1, as shown in Figure 2 and Table 1, of the 11 iliofemoral arteries exposed to ultrasound alone without DDFP, only 1 (9%) was recanalized with TIMI grade 2 flow after 45 min of intervention. The other 10 arteries remained occluded after 60 min of the treatment phase. In contrast, of the 11 iliofemoral arteries treated by combination of transcutaneous ultrasound and DDFP, TIMI grade 3 flow was achieved in 7 (64%), TIMI grade 2 flow in 1 (9%) and TIMI grade 1 flow in 1 (9%). TIMI grade 3 flow was accomplished in three arteries after 15 min, in two arteries after 45 min and in two arteries after 60 min of intervention. TIMI grade 2 and 1 flow was attained after 30 and 60 min of intervention, respectively. As shown in Table 1, TIMI grade 3 flow, more than TIMI grade 2 and 1 flow, was achieved with significantly higher frequency (64% vs. 0%, $p = 0.0039$; 73% vs. 9%, $p = 0.0075$; 82% vs. 9%, $p = 0.0019$, respectively) in the iliofemoral arteries treated by combination of transcutaneous ultrasound and DDFP than in those treated by ultrasound alone.

In protocol 2, as shown in Figure 3 and Table 1, no artery was recanalized at the end of the protocol among six occluded

Table 1. Frequency of Thrombolysis in Myocardial Infarction Grade 3, 2 and 1 Flow Achieved in Protocols 1 and 2

Protocol 1: Transcutaneous Ultrasound Exposure Alone and Transcutaneous Ultrasound+DDFP			
TIMI Flow Grade	Ultrasound Alone	Ultrasound +DDFP	p Value
3	0/11 (0%)	7/11 (64%)	0.0039
≥2	1/11 (9%)	8/11 (73%)	0.0075
≥1	1/11 (9%)	9/11 (82%)	0.0019
Protocol 2: DDFP Alone and Transcutaneous Ultrasound+DDFP			
TIMI Flow Grade	DDFP Alone	Ultrasound +DDFP	p Value
3	0/6 (0%)	4/6 (67%)	0.0606
≥2	0/6 (0%)	5/6 (83%)	0.0152
≥1	0/6 (0%)	5/6 (83%)	0.0152

Protocols 1 and 2: Overall Frequency of TIMI Grade 3, 2 and 1 Flow Achieved				
TIMI Flow Grade	Ultrasound Alone	DDFP Alone	Ultrasound +DDFP	p Value
3	0/6 (0%)	0/11 (0%)	11/17 (65%)	0.0003
≥2	0/6 (0%)	1/11 (9%)	13/17 (76%)	0.0001
≥1	0/6 (0%)	1/11 (9%)	14/17 (82%)	< 0.0001

Data presented are number (%) of rabbit arteries. DDFP = dodecafluoropentane emulsion; TIMI = Thrombolysis in Myocardial Infarction.

arteries treated only by DDFP administration. Among six occluded arteries treated by combination of transcutaneous ultrasound and DDFP, four (67%) were recanalized with TIMI grade 3 flow, one (17%) with TIMI grade 2 flow, and only a single artery remained occluded. TIMI grade 3 flow was achieved after 15 min of intervention in one artery, after 45 min of intervention in two arteries and after 60 min of intervention in one artery, and TIMI grade 2 flow was attained after 60 min of intervention. The frequency of TIMI grade 3 flow was not statistically different (67% vs. 0%, p = 0.0606) between the two groups. Greater than or equal to TIMI grade 2 flow was achieved with significantly higher frequency (83% vs. 0%, p = 0.0152) in the iliofemoral arteries treated by combination of transcutaneous ultrasound and DDFP than in those treated by DDFP only.

Figure 4 (A and C) show examples of bilateral rabbit iliofemoral thrombotic occlusions after electrical induction. The left iliofemoral artery treated with ultrasound and DDFP is widely patent (Fig. 4B), whereas the contralateral artery treated with ultrasound alone remains occluded (Fig. 4D). When data from protocols 1 and 2 were combined, as shown in Table 1, recanalization of thrombotically occluded iliofemoral arteries was not achieved even in a single artery by administration of DDFP alone, was successful in 1 (9%) of 11 arteries (TIMI grade 2 flow) treated by ultrasound alone and was accomplished in 14 (82%) of 17 arteries (11 with TIMI grade 3 flow, 2 with TIMI grade 2 flow, 1 with TIMI grade 1 flow) treated by ultrasound and DDFP combined.

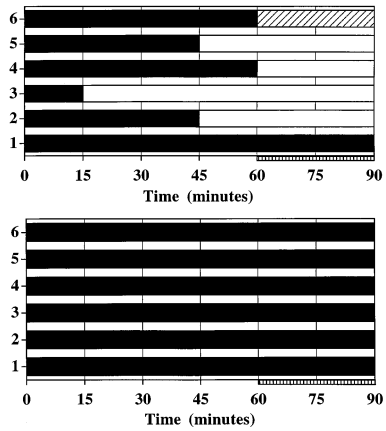

Figure 3. TIMI flow grade of arteries treated by ultrasound exposure and DDFP administration (**top**) and by DDFP administration alone (**bottom**). Of the six iliofemoral arteries treated by combination of transcutaneous ultrasound and DDFP, five were recanalized (TIMI grade 3 flow in four, TIMI grade 2 flow in one). No artery was recanalized after DDFP administration alone. Symbols as in Figure 1.

In both protocols 1 and 2, no angiographic reocclusion was detected with serial angiographic monitoring 15 and 30 min after termination of the intervention. Moreover, no distal or side branch embolization was observed after successful arterial recanalization.

Tissue temperature. Tissue temperature of the soft tissue around the occluded artery was 30.0 ± 0.3°C before and did not change at 5 min, but increased up to 42.6 ± 0.9°C at 10 min of ultrasound exposure without cooling procedures. Skin temperature rose to 63.8 ± 2.9°C at 5 min and up to 73.3 ± 4.7°C at 10 min of ultrasound treatment.

Histopathologic results. Figure 5A shows a patent vessel with only minimal focal residual mural thrombus after ultrasound and DDFP exposure. Figure 5B is an example of a thrombotic occlusion in an iliofemoral artery that received ultrasound but not DDFP. The 10 arteries that were patent by angiography were also patent by microscopy. In these patent arteries, four had no thrombus, two had mild mural residual thrombus, three had 10% to 20% residual thrombus, and one had <50% residual thrombus. Mild focal necrosis of the vessel wall was noted in four arteries. More severe histopathologic changes were found in thrombotically occluded arteries (25% to 50% of vessel wall necrosis with mural thrombus), presumably because of longer duration of ultrasound exposure. The rabbit dermis as well as subcutaneous soft tissue at the site of ultrasound exposure revealed thermal damage characterized by coagulation of the tissue (necrosis) with focal hemorrhage.

Discussion

This study demonstrates that the clot-disrupting effect of transcutaneous ultrasound was significantly enhanced by DDFP microbubbles in both in vitro and in vivo experiments

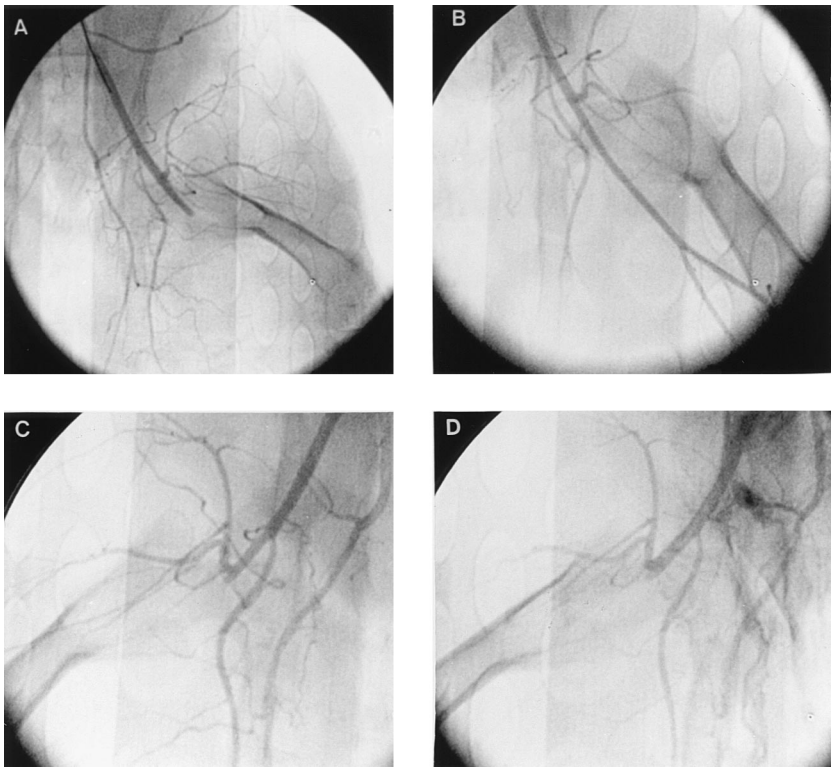

Figure 4. Angiographic example in which the left iliofemoral artery was treated by transcutaneous ultrasound and DDFP. **A and C,** Baseline angiographic thrombotic occlusions of both iliofemoral arteries. **B,** Angiogram of the widely patent left iliofemoral artery after 30 min of treatment with transcutaneous ultrasound and DDFP. **D,** Angiogram of the occluded right iliofemoral artery, which received 60 min of treatment with ultrasound alone.

without a thrombolytic agent. The simple combination of transcutaneous ultrasound and DDFP emulsion has potential for clinical application in patients with thrombotic arterial occlusions.

Transcutaneous ultrasound clot disruption without thrombolytic agent. There are currently two major areas of research in ultrasound clot disruption: catheter-delivered high intensity, low frequency (17.5 to 27.5 kHz) ultrasound without concomitant use of a thrombolytic agent (9–17) and external (remote) exposure of higher frequency (170 kHz to 3.4 MHz) ultrasound in combination with a thrombolytic agent (18–25,27,28). Catheter-delivered ultrasound was successful in dissolving clots in vitro (9–15), in vivo in animal models (12,14,16) and in humans (16,17) without concomitant use of thrombolytic agents. In contrast, most of the external ultrasound clot dissolution studies have been performed using ultrasound at higher frequencies (170 kHz to 3.4 MHz) and have demonstrated enhancement of drug-induced fibrinolysis in vitro (18–25,27,39) and in vivo in animal models (19,28–30). Only three in vitro studies (26,27,31) reported the efficacy of low frequency (20 and 26 kHz) ultrasound clot disruption without a thrombolytic agent. Our in vitro data in the present study confirm the results of these reports showing significantly greater reduction in clot weight after ultrasound exposures at 24.8 kHz compared with the control sample without ultrasound exposure. Furthermore, in vitro the clot-disrupting effect of ultrasound was markedly enhanced by DDFP. In the in vivo portion of our study, transcutaneous ultrasound alone was able to recanalize only 1 of 11 thrombotically occluded arteries. The discrepancy between our in vitro and in vivo studies might be due to the difference in ultrasound intensity at the site of thrombus. In the in vivo study, the ultrasound intensity might have declined to a lower level that was not sufficient to disrupt thrombus by itself. However, when combined with DDFP administration, transcutaneous ultrasound recanalized occluded rabbit iliofemoral arteries with TIMI grade 3 flow with a significantly higher frequency (0 of 11 vs. 7 of 11, $p = 0.0039$).

Mechanism of external (transcutaneous) low frequency ultrasound clot disruption and effect of echo contrast agents. The predominant mechanism of external (transcutaneous) low frequency ultrasound clot disruption is believed to be acoustic cavitation (unstable or transient cavitation) and precavitation (stable cavitation) as well as the contribution of microstreaming and microcurrents generated under an acoustic pressure field (9,10,13–15,19–21,26–32). *Cavitation* (unstable or transient cavitation) is the formation and collapse of microscopic bubbles when ultrasound waves pass through a liquid with an alternating pressure. These bubbles, or cavities, take some cycles to grow and then collapse violently at the point called *resonant size*, which is larger at lower frequencies. The collapse of larger bubbles generates more energy than smaller bubbles. *Precavitation* (stable cavitation) is the oscillation or vibration of microbubbles present or formed in the acoustic field. It generates large shock waves and also causes microstreaming of the liquid. If microbubbles of DDFP already exist around the clot, it can be assumed that they will oscillate and vibrate as well as grow and collapse, generating high energy and causing clot disruption.

Tachibana and Tachibana (39) demonstrated enhancement of the thrombolytic effect of urokinase by initial treatment

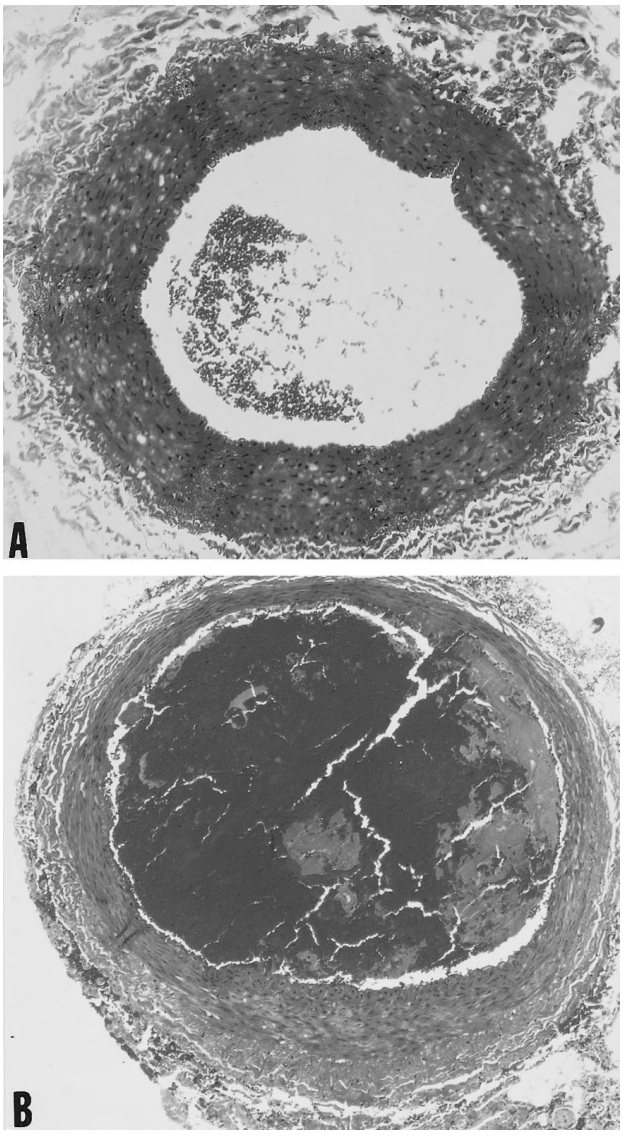

Figure 5. Histologic sections of rabbit iliofemoral arteries. **A,** Rabbit iliofemoral artery that was angiographically patent after 30 min of treatment with transcutaneous ultrasound and DDFP; the artery is patent and without evidence of wall damage. **B,** Thrombotic occlusion of a control iliofemoral artery not receiving DDFP. Hemotoxylin-eosin stain. ×10, reduced by 26%.

using sonicated human serum albumin microbubbles and ultrasound. However, the experimental setting is different from our in vitro studies; ultrasound at a frequency of 170 kHz was irradiated in pulsed mode for 3 min with continuously infused sonicated human serum albumin microbubbles at the beginning of the study, and the thrombolytic effect of urokinase was examined for 30 to 120 min. Recently Porter et al. (27) reported that a 20-kHz, 1.5-W/cm^2 ultrasound system and perfluorocarbon-exposed sonicated dextrose albumin, which is also an echo contrast agent, resulted in enhanced ultrasound clot disruption, from 24% with ultrasound alone to 43%. Although the agent, perfluorocarbon-exposed sonicated dextrose albumin, is different from DDFP, these findings are similar to our in vitro studies.

Clinical significance. This in vitro and in vivo study suggests the possibility for nonpharmacologic clot disruption in patients with peripheral and coronary arterial thrombotic occlusions using transcutaneous exposure of ultrasound energy in combination with DDFP administration. This technique might also enhance drug-induced fibrinolysis (although this hypothesis was not tested in the present study), resulting in more effective and rapid thrombolysis or reduction of hemorrhagic complications by a decreased dosage of thrombolytic agent needed.

Study limitations. A number of limitations have to be overcome before clinical application of this technique. Transcutaneous application of high intensity, low frequency ultrasound was associated with a varied extent of injury to the rabbit skin and subcutaneous tissue. This side effect is probably caused by direct thermal effect of ultrasound, indirect thermal effect through the ultrasound transducer and partially by cavitation of ultrasound gel placed between the transducer and the skin. To reduce this complication of ultrasound, some appropriate cooling system might be needed. Another possibility is to decrease the intensity or increase the frequency of ultrasound or establish some method to enhance the clot-disrupting effect of ultrasound (e.g., the combination of a thrombolytic agent and DDFP). The application method of ultrasound (attachment of transducer to the skin through ultrasound gel) might also need modification.

In the clinical setting, there is a substantial distance between the clot and ultrasound probe due to the intervening tissue compared with the experimental setting using a rabbit model. The intensity of ultrasound energy might not be enough to disrupt the clot in that situation. To apply ultrasound energy noninvasively, the exact location of the clot will have to be identified by ultrasound or other technique. In our experiment, DDFP was directly injected into the iliac artery. However, it might be very difficult to concentrate a sufficient amount of DDFP microbubbles around thrombi by intravenous injection without catheterization. To accomplish this, a tissue (thrombus)-targeted contrast agent, as reported by Lanza et al. (40), might be worth utilizing. DDFP is reported to cause negative hemodynamic effects in canine models (35); however, this unfavorable effect has not been reported to be significant in monkeys (37) and humans (38). Another potential side effect of ultrasound irradiation is the activation of platelets (41), which might cause reocclusion of the artery. In a rabbit model, it was reported (29) that ultrasound at 1 MHz and 6.3 W/cm^2 significantly shortened the time to initial reflow but was associated with worse overall arterial patency and more frequent arterial reocclusion after initial recanalization. However, in our study, no reocclusion of the recanalized artery was observed at 30 min, and in a canine model, ultrasound at 200 kHz and 0.25 W was reported (42) to be useful for the prevention of reocclusion after successful thrombolysis of the femoral arteries by recombinant tissue-type plasminogen activator. Moreover, in human trials of ultrasound angioplasty using a frequency of 20 kHz, there has been no evidence to suggest a prothrombotic effect in peripheral or coronary

arteries; rather, effective thrombus disruption has been seen (17,43–45).

Conclusions. This study demonstrates that low frequency ultrasound dissolves a substantial amount of clot without a thrombolytic agent in vitro, and DDFP microbubbles markedly enhance ultrasound clot disruption. In the in vivo rabbit iliofemoral thrombotic occlusion model, arterial recanalization was accomplished significantly ($p < 0.01$) more often with the combination of transcutaneous ultrasound and DDFP emulsion than by transcutaneous ultrasound alone or DDFP emulsion alone. These findings suggest that the simple combination of transcutaneous ultrasound and DDFP emulsion without a thrombolytic agent has potential for clinical application in patients with thrombotic arterial occlusions.

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